THE TESTING OF GERMICIDAL SUBSTANCES AGAINST THE GONOCOCCUS

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In gonorrhea, a disease second to none in frequency and economic and social importance, the treatment is almost entirely empiric. The literature contains but few experimental studies on this subject, and the few to be found are incomplete and inconclusive. In undertaking a series of laboratory researches on venereal disease, we have attempted, by experimentation, to lay down some principles on which therapy can be based and, if possible, eventually to attain a rational basis in this field comparable to that in the other fields of medical treatment.

In gonorrhea in the male, one is dealing, in the majority of cases, with a local infection beginning on a mucous surface and extending into the deeper tissues and glands, but remaining confined to the organs immediately contiguous to the original site of infection. The anatomic structure of these organs bears an important relation to the course and outcome of the disease. The urethra is surrounded, from the fossa navicularis to the vesical orifice, by various glandular structures, of which the glands of Littre, the glands of Cowper, and the prostate gland are the most important. All of these glands are racemose, convoluted and of narrow lumen. The gonococcus penetrates them readily, and in a short time they become plugged with exudate or closed off by scar tissue. As a part of the researches in this laboratory, a study has been made of the anatomy of these glands in the embryo and in the adult [1] and an examination of Johnson's plates shows what a labyrinth of passages exists, and how deeply the anatomic relations must influence the clinical course of a gonorrheal urethritis.

The gonococcus calls forth a cellular exudate made up, in the early stages, mostly of polymorphonuclear leukocytes. The organism undergoes phagocytosis by these leukocytes, but it is characteristic that only a small proportion of the cells in a given exudate ingest the

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[1] Johnson, F. R., Anat. Record, in press.

gonococci, and even in these there is evidence to show that the intracellular cocci remain viable.[2] The reaction, then, by which the body seeks to overcome the gonorrheal infection, is not entirely a phagocytic one. Work with vaccines has not shown that a bactericidal immune body is effective in curing urethral infections. It is possible that the rapid development of scar tissue creates walled-off nests of infection, for example, in Littre's glands or in the prostate, where the immune forces of the body are unable to penetrate. Extensive microscopic studies of acute gonorrhea will undoubtedly throw much light on these problems. Material is now being collected in this laboratory for such study.

Gonorrhea, unlike many other infections, occurs in a locality in which it may be subjected to direct treatment with antiseptic or germicidal drugs. It is self-evident that treatment should be given at an early stage whenever possible, before the infection has embedded itself deep in the peri-urethral structures. Therapy with drugs based on the conception of their germicidal action, has had a fair measure of success. Other things being equal, drugs capable of being put in true solution and having high penetrating powers, such as those of the mercurochrome series developed in this laboratory,[3] should be more effective in reaching all the foci of infection. The first step, therefore, to be made in solving the problem of gonorrhea is to obtain reliable data concerning the germicidal value of drugs for gonococci when brought in contact with them, in order to determine what parallelism exists between germicidal activity and therapeutic effect.

PREVIOUS METHODS

A number of investigators have sought for methods of determining the efficiency of drugs against the gonococcus, and all have encountered difficulties in their work. These difficulties have resulted mainly from the uncertain and unsatisfactory growths of the gonococcus on culture mediums. This obstacle has only recently been overcome, and the reader is referred to the article by Swartz [4] in which a full discussion of recent and older culture methods will be found.

[2] Scholtz, W., Arch. f. Dermat. u. Syph., 1899, 49, p. 3; Deut. med. Wchnschr., 1905, 31, p. 935; Neisser u. Scholtz, Handb. d. path. Mikroorg., 1903, 3, p. 148.

[3] Young, H. H.; White, G C., and Swartz, E. O.: Jour. Am. Med. Assn., 1919, 73, p 1483.

[4] Jour. Urology, 1920, 4, p. 325.

This difficulty in obtaining large amounts and reliable growths of gonococcus has led some of those working on this problem to adopt methods based on what may be called the "antiseptic" or "inhibition" principle. Quantities of the drug to be tested are mixed with the culture medium in such a manner that known dilutions thereof are present in the successive tubes of a series varying from weak to strong. A heavy implantation of gonococci is then made in each tube, and all are cultivated in the incubator in the usual manner. The criticism of this method is that the drug acts on the organism during the entire period of culture, a condition that bears no relation to clinical use in which the drug is effective for only a limited time. Further, the presence of a constant concentration of the drug in the tube may inhibit all growth of the organisms when many or all have escaped actual killing and remain quite viable and capable of normal growth were the antiseptic substance removed. This, of course, would mean that such inhibited organisms in gonorrhea would soon multiply and reestablish the infection in the urethra; for here the drug would be removed by absorption, by dilution with exudate, by being chemically broken down, or by flushing of the urethra with urine. Therefore, it is readily seen that this method gives erroneous results, indicating germicidal powers which are too high, and that useful conclusions cannot be drawn from such tests.

The use of this method, with many of the older uncertain culture methods, adds a further element of unreliability to the results so obtained.

As is well known, the principles on which modern germicidal testing is based were enunciated by Geppert [5] and subsequently developed by Kronig and Paul,[6] Rideal and Walker,[7] Madsen and Nyman,[8] Chick and Martin [9] and others.[10] In essence, the methods of these workers consist in placing a quantity of the organisms, usually in the form of a constant or fairly constant emulsion, in a known dilution of the drug to be tested. After standing a definite length of time, a small portion, usually that adhering to a platinum loop of constant size (or 0.1 c c removed by a small pipet) is transferred to a fresh tube

[5] Deutsch. med. Wchnschr., 1891, 17, p. 797.
[6] Ztschr. f. Hyg. u. Infektionskr., 1907, 25, p. 1.
[7] Jour. Roy. Sanit. Inst., 1903, 24, p. 424.
[8] Ztschr. f. Hyg. u. Infektionskr., 1907, 57, p. 388.
[9] Jour. Hyg., 1908, 8, p. 564.
[10] Norton, J. F., and Hsu, P. H., Jour. Infect. Dis., 1916, 18, p. 180. Somerville and Walker, Pub. Health, 1905, 18, p. 385.

of culture medium which is usually plated. The small quantity of drug transferred with the organisms is supposed to be so greatly diluted in the fresh medium that it has no further action. If no growth occurs, one assumes that the organisms were killed during their contact with the solution of germicidal substance. If the gonococcus is employed in this manner, the quantity of organisms transferred to the fresh tube or medium is so small that growth is uncertain and unreliable. This is especially true with the older culture methods in which growth was uncertain under the best conditions. It has been noted that a heavy planting with gonococcus is necessary to obtain the most luxuriant growths.[4]

Steinschneider and Shäffer[11] used a mixture of two parts water and one part human serum, previously heated one-half hour to 60 C., for making their emulsions of gonococci. The emulsion was heavy, and into 2 c c of it was placed the test drug in such a manner that the desired concentration was reached. The mixture was kept at 35° C. and at the end of the desired time 3 loopfuls were removed and planted on serum agar. By this method, an appreciable quantity of drug must have been carried over to the culture medium, and the number of gonococci transferred must have been comparatively small. Few of the drugs in the series of Steinschneider and Shäffer are now in use, but in cases in which comparison can be made, their figures are not greatly different from ours. Their work is by far the best on this subject up to the present time.

Post and Nicoll[12] tested many germicides by a method in which one loopful of the organism was placed in 0.5 c c of a solution of the test drug. At the end of 1, 5, 10 and 30 minutes, and 20 hours, 1 loopful of the drug-organism mixture was transferred to blood agar and plated. The temperature of the reaction is not stated. The results show in the main low germicidal activity for the drugs tested. Argyrol, 50%, failed to kill in 30 minutes, and the gonococcus is said to grow well after suspension in distilled water for 20 hours. No mention is made of microscopic controls of the final growths. These results are at variance with the experience of ourselves and others with this organism.

Clark and Wylie[13] mixed 2 c c of a salt solution emulsion of gonococcus with 2 c c of a solution of the test drug. At the end of 5, 15 and 30 minutes, one loopful was transferred to ascitic fluid agar and plated. The temperature of the reaction was not stated. They tested a series of silver compounds and cresol and found the silver compounds comparatively ineffective. The method and checks are not described in great detail.

Furstenau[14] used the inhibition method and also a method in which the drug was added to broth cultures of gonococcus. Transfer to the culture medium was made with a platinum loop. The temperature at which the tests were carried out is not stated. The results of this author with acriflavine show high killing power, which cannot be reconciled with any of our results.

[11] Kongress Deutsch. Dermat. Gesellschaft, Breslau, 1894, p. 156.
[12] Jour. Am. Med. Assn., 1910, 55, p. 1635.
[13] Ibid., 1911, 57, p. 394.
[14] Ztschr. f. Augenheilk., 1918, 40, p. 1.

Attempts have been made to modify the technic to make it more suitable for gonococci. Culver[15] mixed emulsions of gonococci with dilutions of the test drug and transferred a drop of the mixture to slanted mediums. He worked with only four silver compounds.

Steinschneider and Schäffer,[11] as a variant on the previously described method, took agar slants on which growths of gonococcus had been produced, and poured dilutions of the test drug directly on them. After a given time it was poured off, and a portion of the growth remaining on the agar was transferred to a new tube by means of a loop. Few figures for results by this technic are given. so that one cannot conclude how satisfactory the method was found.

DISCUSSION OF PRINCIPLES INVOLVED IN THE GERMICIDAL METHOD

Chick[16] and Chick and Martin[9] have expounded with great clearness the principles underlying germicidal action. All who have tested germicidal substances[17] find that there are great differences in the resistance of various organisms to these agents. It is therefore impossible to postulate from figures hitherto obtained with other organisms, what will be the reaction of a highly parasitic organism like the gonococcus. At the beginning of our work, we felt convinced that in order to avoid the troubles of earlier workers our method must differ from theirs in that comparatively large numbers of gonococci must be exposed to the action of the test drug, and that at the end of the test period a comparatively large proportion of these treated organisms must be transferred to the medium. In order to accomplish the latter object without carrying over too much of the test drug, the cocci would have to be freed from it in some manner. The use of the centrifuge, as first proposed by Schäffer,[18] suggested itself for this purpose.

Chick[16] has worked out the mathematical formulas for disinfection where time, concentration of test durg, or temperature is made to vary. She has also shown that the time of disinfection is a function of the number of bacteria present, following laws exactly similar to those of a chemical reaction, i. e., mass action. A definite proportion of the living organisms is killed in each unit of time. The curve is a hyperbolic one. In tests made on the gonococcus, with a view to a possible therapeutic application, the conditions should be so arranged that only the concentration of the test drug varies. All other factors should be constant or as nearly constant as possible.

[15] Jour. Lab. & Clin. Med. 1918, 3, p. 487.

[16] Jour. Hyg., 1908, 8, p. 92.

[17] Ztschr. f. Hyg. u. Infektionskr., 1907, 25, p. 1; 57, p. 388. Jour. Roy. Sanit. Inst., 1903, 24, p. 424. Jour. Hyg., 1908, 8, p. 654. Kongress Deutsch. Dermat. Gesselschaft, Breslau 1894, p. 156. Kenwood and Hewlett, Public Health, 1906, 18, p. 462. Derby, G. S., Boston Med. & Surg. Jour., 1906, 155, p. 341.

[18] Ztschr. f. Hyg. u. Infektionskr., 1894, 16, p. 189.

Temperature.—The temperature at which the drug is allowed to act on the organisms should undoubtedly be that of the body, 37.5 C.

Time.—As regards the length of time during which this contact continues: We have selected 20 minutes, since we feel that this is the longest time it may conservatively be assumed that any drug acts in gonorrheal treatment, and that in the urethra it undoubtedly acts for this length of time unless a flushing with urine occurs, even when the meatus is not held closed more than 5 minutes. It is our conviction, since time is such an important factor in germicidal action, that any drug intended to kill gonococci in the urethra should be allowed to act for the longest possible time.

Number of Organisms in the Emulsion.—The criteria for constancy in the number of organisms used were similar to those of Chick.[16] Cultures of equal age were used grown on medium always prepared in the same manner and emulsified in the same way with the same quantity of 0.85% sterile salt solution. The age of the strain is of minor importance, for our results with old laboratory strains and with freshly isolated strains were the same. It would undoubtedly be more accurate to count the gonococci in the emulsion. This, however, is difficult and time consuming, owing to the tendency of the organisms to cohere, and since our tests checked well on different days, we did not consider it essential to do so. As stated, the emulsions to be used contained a large number of gonococci. This necessarily means that complete disinfection in a given time will require a higher concentration of the test drug than if a small number of organisms were used in the emulsion. But, as indicated above, this fact, if kept well in mind, does not affect the usefulness of the test in any way.

Number of Organisms Transferred.—The use of the centrifuge enables one to wash the organisms free of the test drug at the end of the stated period, and at the same time, by concentrating them at the bottom of the centrifuge tube, makes it possible to transfer to the culture medium almost all of the organisms originally introduced into the test solutions, instead of say $\frac{1}{3000}$ as with a platinum loop.[19] Chick [16] has shown that with certain germicides, especially those containing the heavy metals, the quantity carried over on a platinum loop to the culture medium may, even when very small, exert an important inhibitory effect if the number of organisms surviving the action of the test drug is small.

[19] Shohl, A. T., and Janney, J. H., Jour. Urology, 1917, 1, p. 211.

Advantages of the Centrifuge Method.—This method is calculated to overcome the objection mentioned above in two ways: First, the organisms are washed free of the test drug as thoroughly as desired, and second, almost all of the organisms in the original emulsion may be transferred to the culture tube. The implantation will still be liberal enough to overcome any small inhibitory effect, even if a considerable proportion of organisms have been killed. For example, let us assume that 10,000,000 organisms are introduced in a test solution which is of such a strength that 99% are killed in 20 minutes. If a loopful, containing $\frac{1}{3000}$ of the mixture be taken, only about 33 living organisms will be transferred. If, on the other hand, by the centrifuge method one transfers 80% of the organisms originally introduced, 80,000 survivors will be planted on the culture tube instead of 33.

Method of Culture After Action of Drug.—After the treated gonococci are transferred to the culture medium, there still remains to be considered the interpretation of the resulting growths. In testing ordinary organisms, it is customary to transfer to melted agar, which is immediately poured into Petri dishes. The number of colonies developing can then be counted in those cases in which some of the organisms have survived. In the case of the gonococcus, there are several objections to this procedure. Since the growth of gonococcus occurs only on the surface of solid mediums,[4] a plate would not indicate truly the number of surviving organisms, but only those which did not come to rest in the depths of the medium. The necessity for using mediums containing uncoagulated human protein would make the pouring of plates, especially in lengthy series of tests, difficult and time consuming. The great susceptibility of the gonococcus to heat, making it necessary to keep agar for the plates nearly at its solidification point, would increase the difficulty. Lastly, any germicidal action falling short of completion would be useless in the urethra. For these reasons, it seems simpler and more expedient to inoculate the treated organisms on slanted solid mediums and to consider as giving positive results only those tubes in which no growth occurs.

The Presence of Protein.—The presence of some protein material in the drug-organism mixtures is desirable, since in practical use the drugs must always act in the presence of protein. This may be attained by the addition of sterile hydrocele or ascitic fluid or blood serum. If the emulsion is made directly from the protein-containing medium, it will contain about 0.4% hydrocele or ascitic fluid.

Comparison with Other Organisms.—It will readily be seen that a method evolved from these principles will be favorable to the organism rather than to the drug. If, however, it is a method by which end-points for different drugs can be readily obtained and by which reliable and consistent growths are to be had, this is not an objection to its practical use. In order to compare the susceptibility of the gonococcus with that of other organisms, however, one would have to perform other tests using the same technic and using the other organisms in place of the gonococcus, taking care to keep the number of organisms in the emulsion comparable to the number used in the gonococcus emulsions. In case the end-points were too high with this technic, necessitating the use of undesirably concentrated solutions of the test drug, the time of the reaction could be extended as desired, calculating back to a period of twenty minutes by the formula of Chick.[16]

Preliminary Studies.—All of the considerations treated in the last section did not make themselves felt at once in the beginning of our work. Having secured, through the work of Swartz,[4] an eminently satisfactory method of growing gonococci with great profusion and certainty, we turned first to the antiseptic or inhibitory method. Little work was done along this line, as the technic was considered faulty from a theoretical point of view. The results thereof have been rejected. One of us (S) thereupon devised a method, later found to be similar to that utilized by Steinschneider and Shäffer,[11] in which the solution to be tested was poured on a 24-hour slant culture of gonococci. After remaining a given time, it was poured off, the culture washed with 0.85% salt solution, and transfers made to fresh medium. This method also has theoretic objections since the thickness of the layer of gonococci is an unknown quantity. The amount transferred varies, since more or less of the layer of gonococci may be removed by the test fluid or the subsequent washings. The results were inconstant, and the need of a more exact and better controlled method was felt.

It was determined to utilize the gonococci in the form of an emulsion. The emulsion may be prepared from slants or from fluid cultures. We have utilized slants throughout our work because growth of the gonococcus occurs in both fluid and solid mediums on the surface,[4] and a greater number can be procured from the larger surface of the slant. The remaining steps of the technic were developed according to the principles outlined in the preceding section.

DESCRIPTION OF METHOD

Our method is as follows:

1. All the tubes and pipets and all the solutions to be used, except those of the test drug, are sterilized. The emulsion is prepared by introducing 7½ c c 0.85% sterile NaCl solution into a 24-hour culture of gonococcus on slanted ascitic or hydrocele fluid agar.[4] The growth is scraped off into the salt solution, using a platinum needle. The emulsion is poured into a tube containing glass beads; and fitted with a 1 c c pipet running through a cotton stopper. After shaking, it is placed in a water bath at 37.5 C. If more than 7.5 c c are needed, a second or third tube may be prepared in the same way and added to the emulsion. Since gonococci do not survive indefinitely in salt solution, the emulsion is always discarded at the end of one hour and a fresh one substituted. While the dilution is made with physiologic salt solution, a small amount of protein will be taken up from the surface of the medium and the "water of condensation." This is allowed to remain.

2. Beginning with a strong solution of the test drug, which is presumably sterile, dilutions thereof are made with sterile pipets and tubes, using sterile distilled water as a diluent. These dilutions are made of twice the strength in which it is desired to test the drug. One c c of each dilution is placed in a sterile, cotton stoppered, properly labeled, 15 c c centrifuge tube. These centrifuge tubes are warmed to 37.5 C. before inoculation.

3. Into each tube is put 1 c c of the gonococcus emulsion. This makes the total contents of the tube 2 c c and reduces the test drug to the desired concentration. The tube is then placed in the water bath at 37.5 C. and allowed to stand 20 minutes. It is our custom to inoculate the tubes at 5-minute intervals. This allows time, at the end of the test period for each tube, to centrifuge, wash, and transfer.

4. At the end of 18 minutes, the tube is removed from the water bath, placed in a warm metal holder, and centrifuged rapidly for 2 minutes in a high-power centrifuge. At the end of 2 minutes the gonococci will be found to have settled to the bottom of the tube much more completely than is the case with, for example, B. coli or Staphylococcus aureus, and the supernatant fluid will be quite clear. This is poured off, replaced by 2 c c of warm 0.85% NaCl solution, the gonococcus thoroughly mixed with the salt solution, and the whole

centrifuged a second time. We usually wash once, but with the more concentrated test solutions, we wash two or three times. One may wash as often as desired.

5. After the final centrifugalization, the last wash fluid is poured off, the gonococci remaining as a compact mass in the tip of the tube. With a properly shaped loop, they can be removed almost completely, since they show a tendency to cohere. Some drugs destroy this tendency to a certain extent, but in any case, a liberal planting can be procured. The organisms so obtained are spread over the surface of a fresh slant of ascitic or hydrocele fluid agar, which is then heated, corked and incubated according to the technic described by Swartz.[4]

6. The tubes are read at the end of 24 hours, 48 hours, and 4 days. Growth usually appears in 24 hours, but with some drugs it may be delayed in some tubes one or two days. All growths are counted as positive, regardless of the number of colonies present. The growth in all tubes is examined in a smear by means of a Gram stain, to detect the possible presence of contamination.

7. At the end of 7 days, all tubes showing no growth are reinoculated from a fresh, viable culture of gonococcus. Each tube should show a growth after this planting, proving that not enough of the test drug was carried over with the transfer loop to make the culture medium unsuitable for growth.

We have utilized this method in testing a large series of drugs. We have found that duplicate tests on different days check within reasonable limits of experimental error. The end-point is sharp and definite. Suitable controls included in each series have always shown profuse growth, proving that the manipulations involved in the method have no deleterious influence on the gonococcus. The method is satisfactory as regards contamination. In spite of the fact that the solutions of the test drug are not sterilized, we have had not more than 7 contaminated series, or about 3.5% of the entire number. All the final growths have been carefully examined microscopically. We have not tested any drugs which precipitate protein, since the results of such tests would be of little value, and we can see no reason for the use of such drugs for their germicidal value.

DISCUSSION

The results of tests made by this method will give definite figures bearing on the ability of various drugs to kill the gonococcus when

brought in contact with it. Such figures will show that certain drugs used in the treatment of gonorrhea do not destroy gonococci. Their beneficial effects are exercised through other means. The figures will also show which of the drugs used or proposed for therapeutic purposes is the most efficient germicide for gonococcus. Trials will determine the exact relation between these laboratory tests and clinical effectiveness.

It is obvious that the effective strength of any drug in the urethra will depend not only on the concentration in which it will kill gonococci, but also on the strength in which it may be used in the urethra without irritation. This relation may be expressed as a factor obtained by dividing the concentration in which a given drug will kill gonococci in a given time by the concentration in which it may be used in the urethra. Thus, if a drug will kill in a concentration of 1 : 20,000 and may be used in a concentration of 1 : 100, the factor will be 200, while another drug which kills at 1 : 40,000, but will be irritating in any strength over 1 : 1,000 has a factor of only 40.

While we cannot at the present time predict the clinical efficiency of a given drug from laboratory tests, it is quite possible that at a future date we will be able to do so. The development of a reliable method such as is described in this paper is a necessary and important step forward toward this goal.

At present, we are in a position to state that the relative efficiency of the drugs commonly used in the treatment of gonorrhea as germicides, is quite different from that commonly supposed, and also that certain organic mercurial drugs tested in this laboratory, which are colorless, water soluble, penetrating, comparatively nontoxic, and do not coagulate protein, are more efficient germicides for the gonococcus than any others tested.

The results of our tests using the method herein described are being published in a series of papers.

SUMMARY

The application of the principles of germicidal action to tests using the gonococcus as the test organism has been discussed.

A new, practical and reliable method for making germicidal tests with the gonococcus as the test organism has been presented.